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Effects of x-irradiation on adults and larvae of Hymenolepis nana (Von Siebold, 1852) Blanchard, 1892 (Cestoda: Cyclophyllidea)

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EFFECTS OF X-IRRADIATION ON ADULTS AND LARVAE OF *HYMENOLEPIS NANA* (VON SIEBOLD, 1852) BLANCHARD, 1892 (CESTODA: CYCLOPHYLLIDEA)

by

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INTRODUCTION

Since 1852, when von Siebold first described the cestode now known as Hymenolepis nana, the unique characteristics of this worm have attracted much attention. Much of the literature dealing with the species involves host-specificity, life cycle studies, host-parasite relationships, and taxonomic studies. Grassi (1887) reported that a single host may harbor both cysticercoids and adults of this tapeworm, a characteristic not known to occur in any other cestode. It might be said that with Grassi began a period of investigation, disagreement, and conflicting reports insofar as this tapeworm is concerned. Seeking to discover an intermediate invertebrate host, whereby an indirect life cycle could be accomplished, Grassi failed in all attempts to infect several species of arthropods and mollusks. It remained for Bacigalupo (1928) to discover that H. nana may develop in either of two ways: directly without use of an intermediate host, or by an indirect cycle involving an intermediate host.

Grassi (1887) also initiated an even more complex path of investigation into the nature and identity of this worm. He fed to a child mature eggs of a worm obtained from a rat, but was unable to infect the child. On the basis of Grassi's findings, Blanchard (1891) considered the cestode parasitizing man as H. nana, and that parasitizing mice as H. murina, the latter species originally named by Dujardin (1845).

Stiles (1906), however, considered these two cestodes as a single species and believed the mouse-inhabiting form to be a variety of the species found in man, calling it fraterna. Joyeux (1920) after experimentation, stated definitely that the cestode of man and that of rat are not sufficiently similar to be considered as varieties, but that they should be considered as distinct biological species. Chandler (1922) also failed to infect rats with eggs of H. nana obtained from man. Saeki (1920) fed eggs from a human source to mice and obtained positive results; he later fed to a child eggs of a rodent source and again obtained positive results. Uchimura (1923) confirmed Saeki's work and stated that the species are indistinguishable in all respects. Bacigalupo (1928), however, accepted the dualist theory, basing his belief that two species are involved on the differing rates of development in an intermediate host of cysticercoids from the two sources.

The exact nature of H. nana, as a species, is not entirely agreed upon by all investigators, although many are of the opinion that the worms from man and those from mice and rats are one and the same. In the studies herein reported they are considered as such.

Difficulties in establishing the identity of the worm to the satisfaction of all investigators has, I believe, hampered research on other aspects of Hymenolepis. Little work has been done on the histology, physiology, antigenic and other biochemical relationships of the species,

or on the effects which various experimentally-induced environmental conditions may produce.

The importance of animal parasitism has led to many studies on the effects of irradiation on the eggs, larvae, and adults of many parasites. Thus, Trichinella spiralis (Owen, 1935) Railliet, 1895 has been intensively studied as to the effects of radium emanations, x-rays, gamma radiation of radio-active cobalt, and radio-active phosphorus, both in the adult and juvenile stages. Many of the results are conflicting, but much valuable information has been gathered. As for other parasites, there are numerous references to irradiation studies involving protozoa and roundworms, using sunlight, ultra-violet rays or x-rays as sources of irradiation. Among the platyhelminthes, however, very little work has been done. Fastier (1948) demonstrated the effect of ultra-violet light upon the viability of the scolices of the hydatid cyst. More recently, Schiller (1957) used x-rays as a mechanism for facilitating the study of morphological variations in Hymenolepis nana.

I have undertaken a study of some effects of x-irradiation on the cestode Hymenolepis nana. This study will attempt to show: (a) an easy method of feeding larval stages to a definitive host and of predicting the number of adults which will develop, (b) a practical and efficient method of obtaining cysticercoids, (c) a simple method of x-raying cysticercoids so that an accurate statement of actual irradiation received can be made,

HISTORICAL

The genus Hymenolepis was created by Weinland (1858) when he formulated a new classification of the family Taeniidae. Fuhrmann (1907) undertook a general classification of the order Cyclophyllidea and established the family Hymenolepididae. Members of the genus Hymenolepis are characterized by having a small, filiform body, small scolex provided with a well-developed retractile rostellum and armed with a simple corona of 24-30 hooks. The neck is long; the proglottids are much wider than long, and are rarely less than 150 in number. Marginal genital pores are present, and the reproductive organs are dorsal with respect to the excretory and nervous systems. The male reproductive system includes three dorsally-located testes in each mature proglottid. Gravid proglottids contain rounded eggs, each containing two membranes widely separated from one another. The inner membrane surrounding each onchosphere has, at each pole, a thickening from which arise four to eight terminal filaments.

The elucidation of the life cycle of Hymenolepis nana was a result of the studies of Grassi (1887). Following long and minute observations, this Italian author suggested the hypothesis that one host may harbor both larval and adult stages of the tapeworm. Bacigalupo (1928a, b) showed that the development of H. nana may take place in two different

and (d) some effects of x-irradiation upon the dwarf tapeworm, Hymenolepis nana.

ways, either with the aid of an intermediate host or directly within the definitive host, a biological fact never before recognized. A resumé of Grassi's experiments on the direct method of the life cycle is as follows: Parasite-free rats were fed mature eggs of H. nana, and 25-50 hours later, freed hexacanth embryos were found in the intestines, embedded in the mucus at the base of the villi. In about three to four days, larvae were completely developed and situated within villi. Scolices then penetrated into the lumen of the intestine, by rupturing the villi, and the necks began to grow; the adult worms attached to the mucosa of the last five to ten centimeters of the small intestine. The worms matured in 15 to 30 days.

Bacigalupo (1928a, b) described the events of the indirect life cycle as follows: After ingestion of the eggs of H. nana by the beetle Tenebrio molitor Linnaeus, 1758, the membranes surrounding the onchosphere are lost in the intestine, thus liberating the embryo. This larval form penetrates the intestinal wall, enters the body cavity (haemocoel) of the coleopteran where it is believed to assume a rounded form. After three to four days the tail is formed, and at the opposite end cellular multiplication begins; a spur appears which marks the site of the future scolex. At this time, the tail is much larger than the anterior formation and already contains the original six embryonic hooks. The portion destined to form the scolex continues its development; the suckers form, followed

by the appearance of the rostellum and of the adult hooks. Invagination proceeds and the mature, infective cysticercoid is formed. Voge and Heyneman (1957) confirmed this sequence of events, describing each phase in histological detail. In such a cycle, development is slow, requiring about 18 days, as contrasted to the much shorter period (three to four days) involved in the direct mode of development noted above. When these cysticercoids are fed to white mice, adult parasites develop. The time involved from the ingestion of viable eggs to the attainment of sexual maturity of adult worms is approximately 15 to 20 days.

The definitive hosts thus far described for H. nana are man, mouse, rat, and, recently, the ground squirrel. Several coleopterans may serve as intermediate hosts, including Tenebrio molitor (mealworm), Tribolium confusum Duval, 1868 (confused flour beetle), Pulex irritans Linnaeus, 1758 (human flea), Xenopsylla cheopis (Rothschild 1903) (tropical rat flea), and Ctenocephalides canis (Curtis 1826) (dog flea).

Since H. nana is unique among cestodes in its life cycle, the host-parasite relationships of this worm are extremely unusual. It is possible to have in a single host a parenteral phase (cysticercoid) and an intestinal lumen phase (adult).

It has been shown by numerous investigators that many helminths, known to be infective for certain host animals, become less infective with increasing age of the host. Such resistance on the part of the host

has been termed age resistance. In the case of the dwarf tapeworm (H. nana), Grassi (1887) found that rats three or four months old were more susceptible to infection than were either older rats or those just weaned. Although both rats and mice show this phenomenon, age resistance develops more rapidly in rats than in mice, according to Shorb (1933). Larsh (1951) stated that two and one-half months is the most susceptible age for infection among mice. Resistance of older animals to infection may indicate that they are able to avoid infection by the development of specific substances (antibodies) produced in the body. These antibodies may be detected by standard serological procedures. Larsh (1942) demonstrated that such protection against H. nana is found in young mice which receive specific antibodies from their mothers. These antibodies are transferred either in utero or in even greater degree in the milk and are elaborated in the body of the mother in response to antigenic stimulation.

Grassi (1887) and Joyeux (1920) reported that when one infestation of H. nana is present in a rat, hyperinfestation is impossible. Shorb (1933) however, reported that hyperinfestation is possible, but that the size of the individual worms resulting from the superimposed infection is much reduced.

In recent years there has been much interest in the field of irradiation and its effects on living systems. The importance of animal

parasitism has led to many studies on the resistance of parasites to different types of rays. One of the earliest experiments in the field of radiation in parasitology was conducted by Tyzzer and Honeij (1917). Before this time, there had been some biological experimentation with radium which was shown to have a pronounced effect on the development of the germ cells of various animal species, but no conclusive interpretation of results can be found in the literature. Tyzzer and Honeij recognized the possibility of using radium for the destruction of certain parasites, and observed that radium might be employed to advantage in the treatment of cases of schistosomiasis in which the bladder is involved. These same authors experimented with the effects of radium on Trichinella spiralis in rats. They determined the lethal dosages for mice, worms, for the juveniles, and the dosage necessary to produce sterility in adult worms.

Schwartz (1921) investigated the influence of x-rays on Trichinella spiralis. He found that encysted trichinae are injured by relatively heavy dosages of x-rays. The injury, though not visible in the encysted juveniles by structural or functional modifications, became apparent only when juveniles reached a suitable host in whose intestine they are normally capable of continuing their development. Trichinae from meat previously exposed to strong dosages of x-rays underwent rapid granular degeneration in the intestine of the host. Furthermore,

he found that encysted juveniles which had been exposed to lower but still injurious dosages of x-rays were able to continue development in the intestine. Such juveniles, however, did not attain structural and functional sexual maturity. The sex cells appeared to be atrophied and no evidence of successful copulation could be found.

More recently, much experimentation, most of which has been done in the last 25 years, has been undertaken to determine the effects of radiations on parasites. A brief review dealing with the nature of some of these experiments follows.

Halberstädter (1914) found that cultures of Trypanosoma brucei Plimmer and Bradford treated with radium lost their infectivity although they remained viable as evidenced by their continued motility. Patel (1936) irradiated cultures of T. brucei with x-rays and ultra-violet light and found the organisms resistant to the former but rapidly destroyed by the latter. Halberstädter (1938) exposed T. gambiense Dutton 1902 to varying dosages of x-rays and found that 600,000 r were required to kill the organisms, although they could be rendered non-infective for mice by doses of 12,000 r. Emmett (1950) exposed T. cruzi Chagas, 1909 to x-rays with dosages varying from 10,000 to 100,000 r, and noted that the trypanosomes remained viable and were morphologically unchanged. Starting with dosages of 10,000 r, a definite decrease in infective power was induced.

Nasset and Kofoid (1928) found large Endamoeba histolytica (Schaudinn, 1903), resulting from continued growth but retarded fission, after exposure to radium. E. histolytica, exposed to gamma rays for 24 to 48 hours, reached a maximum growth one to several days sooner than did controls.

Chickens infected with x-ray-attenuated oocysts of Coccidia were found by Waxler and Herrick (1941) to acquire a certain amount of resistance to a second heavy infection. This resistance was almost as great as that afforded survivors infected originally with untreated oocysts.

Studies of ultra-violet irradiation of cysts of Echinococcus granulosus (Batsch 1786) Rudolphi 1805 at 2537 Å by Fastier (1948) demonstrated slight impairment of scolex vitality in the hydatid cyst. Although a slight reduction occurred in the number of viable scolices after irradiation, there was apparently little latent effect of irradiation. However, after long exposures at a distance of three centimeters, a definite lethal effect was apparent. The low tissue penetration of rays of 2537 Å, the long exposure times involved, and the incomplete scolicidal action would preclude any application of such techniques in cases of human echinococciasis.

By exposing the eggs of Hymenolepis nana to x-irradiation ranging from 5,000 to 40,000 r, Schiller (1957) found that mutations occurred which were directly proportional to the roentgen dosage. These mutations, however, according to Schiller, are the normal variations one

expects to find in nature. The x-rays did not produce additional mutations. These variations occur in varying intensity throughout the strobila and are not limited to one area only. Schiller found that no adults were obtained from eggs which had received more than 30,000 r.

Radiation by radio-active metals is one of the newest fields in irradiation. Alicata and Burr (1949) exposed trichinous rat meat to 12,000 r of gamma radiation of radio-active cobalt. Subsequent feeding experiments indicated that 60 to 100 per cent of the resulting adults were sterile. McCoy, Downing, and Van Voorhis (1941) found that when rats, heavily infected with trichinae 9 to 10 months previously, were fed radio-active phosphorus, a ready exchange of phosphorus ions took place through the cyst wall surrounding the organism in the muscle. This would imply that the juveniles are probably undergoing active metabolism during the encysted stage. Their experiments, however, did not include observations on the effects of radio-active phosphorus upon the reproductive abilities of the parasites.

Extensive studies on the effects of monochromatic ultra-violet irradiation on the pin-worm, Enterobius vermicularis (Linnaeus 1758) Leach, 1853 have been carried on by Hollaender and co-workers (1940). Jones, Jacobs, and Hollaender (1939), using eggs of this parasite in the infective stage, found a slight increase in inactivation at wave length 2805 $\overset{\circ}{A}$, followed by a depression, and an increased sensitivity of the eggs

at wave lengths below 2400 Å after radiating them with ultra-violet. Hollaender, Jones, and Jacobs (1940), continuing the above experiments, undertook to study the quantitative response of the organism to the lethal limits. After ultra-violet irradiation between 2280 and 2950 Å, they concluded that the inhibition of hatching could be produced by: (1) hardening of outside protein layer; (2) a change in the composition of the lipoid membrane; (3) injuring the embryo; (4) production of toxic substances; or (5) a combination of all of these.

Jones, Jacobs, and Hollaender (1940) studied the sub-lethal effect on E. vermicularis after ultra-violet exposure. In this experiment it was found: (1) that the rate of hatch is lower for irradiated eggs than for control eggs; (2) that there is a delayed lethal effect of ultra-violet radiation, since eggs stored after having been irradiated with a considerable amount of energy showed a smaller percentage hatch than others of the same batch which were tested immediately; (3) that many juveniles hatching from irradiated eggs survived for a shorter time than those hatching from eggs in control dishes; and (4) that ultra-violet radiation is more damaging to immature eggs than to those which have attained a further degree of development.

Jones and Hollaender (1944) used the eggs of Enterobius vermicularis (infective stage) and of Ascaris lumbricoides Linnaeus, 1758 (one-cell stage) to study their viability following ultra-violet radiations approxi-

mating that of tropical sunlight. They found a delay in the development of A. lumbricoides, and tests with E. vermicularis indicated a decided temperature relationship, namely that increasing temperature apparently enhanced the effect of irradiation.

Spindler (1940), using the eggs of Ascaris lumbricoides var. suum found that eggs in all stages of development failed to survive comparatively short exposure to direct sunlight. However, eggs in an advanced stage of development were noticeably more resistant than were undeveloped ones.

A similar phenomenon was observed by Wright (1936) in relation to tests on the effects of ultra-violet light on eggs of Toxascaris leonina (v. Linstow, 1902) and Toxocara canis (Werner, 1782). Although most of the eggs in cultures previously exposed to ultra-violet light were killed, a few survived and were able to complete their development.

MATERIALS AND METHODS

Definitive and Intermediate Hosts

Definitive hosts

Two separate colonies of mice were maintained for these studies. A colony of laboratory-reared white mice known to be free from Hymenolepis nana was maintained for experimental feedings. These mice were examined periodically to make certain they carried no cestode infection. Careful records of litter dates were kept and the only white mice employed in feeding experiments were those between two and four months of age.

A second colony of several breeds of laboratory-reared mice known to harbor H. nana was kept as a source for gravid worms. One strain in particular, known as the "P strain (multiple marker)" was found to be especially susceptible to infection by this cestode. Infected mice were checked regularly to ascertain that their worm burden was H. nana.

A commercial laboratory chow was always available to the mice and was kept in wire baskets suspended from the side of plastic animal cages. Water was supplied by an attached bottle. Cages, feeder baskets and water bottles were never transferred between infected and non-infected colonies without first thoroughly washing and drying them. Separate supplies of laboratory chow were kept for infected and non-infected

colonies. After a white mouse was experimentally exposed to eggs or larval stages of H. nana, it was isolated in a clean cage so as to exclude the possibility of coprophagous infection from another source.

Intermediate hosts

Laboratory-reared colonies of the beetle, Tenebrio molitor, were maintained as a source for intermediate hosts for the tapeworm. The beetles were grown in battery jars containing a commercial bran, and the jars were covered with a triple thickness of cheese cloth. Thin slices of apple were placed in the jars periodically to provide the moisture necessary for the maintenance of the colony. The cultures were kept at room temperature. Various stages in the life cycle of this beetle were isolated from one another as they appeared. Adults were kept in one set of jars, the larvae in others, and the pupae maintained in a third series.

Feeding Experiments with Mice

Several experiments were conducted involving the direct cycle of development (i. e., without the use of an intermediate host). These experiments were carried on in an effort to determine effective methods of feeding to laboratory-bred mice a given amount of worm material so as to predict within close range the number of adult worms one might expect to recover. Twenty-four hours before mice were fed, their water

supply was removed. This facilitated subsequent feeding, for the animals readily ingested the suspensions of eggs, proglottids or cysticercoids. The material to be fed was offered in a pipette, and afterwards the pipette was thoroughly rinsed in a small amount of saline, and the suspension checked to determine the actual number of infective agents ingested by a mouse.

Gravid proglottids were tested first. One group of laboratory-reared white mice was fed proglottids immediately after the gravid tapeworms were removed from the intestinal tract of an infected mouse. A second group was fed proglottids which had been incubated at room temperature for 24 to 40 hours, and a third group was fed proglottids previously incubated 12 hours in a refrigerator. Results of these experiments are summarized in Table 1.

Feeding of eggs to laboratory-reared mice involved eggs from two sources. Some were obtained from gravid proglottids, others from fecal material removed from the rectum of an infected mouse. Some of the eggs were fed immediately. Others were incubated at room temperature for 24, 40, or 72 hours before feeding. Results of these experiments are summarized in Table 2.

It soon became apparent that neither eggs nor proglottids, when fed to mice, would give consistent results as to the number of adults which could be expected to develop. Therefore, cysticercoids were used, and

the indirect life cycle undertaken. Cysticercoids were recovered from laboratory-reared Tenebrio molitor and fed to mice, using the same feeding procedures as those described above. Ten days were allowed for development of adult worms in the small intestine. For post-mortem examination, the small intestine was removed, and the contents of the last half of it removed, so that a count of the worms could be made. Results of these experiments are summarized in Table 3. Techniques involving the feeding of cysticercoids demonstrated that approximately 82 per cent of the larvae would develop to maturity.

Feeding Experiments with Beetles

Laboratory-reared beetles, Tenebrio molitor, were fed gravid proglottids by one of two methods. In one type of force-feeding, a single beetle was held relatively immobile, dorsal side down, on modeling clay. A few gravid proglottids held on the end of a dissecting needle were placed on the mouthparts of the beetle, and were quickly ingested. A second method of feeding involved the isolation of one or more beetles in a small container to which several pieces of apple, approximately 1x3x3 centimeters in size, were added. The upper surface of the apple was then completely covered with gravid proglottids of H. nana, and the beetles were allowed to feed at will. It was shown experimentally that 13 days were sufficient for the development of cysticercoids. To recover cysticercoids from a beetle, the wings and legs were removed and the

insect placed in a Syracuse watch glass containing Ringer's insect saline. The beetle was decapitated, the dorsal body wall slit open, the contents of the haemocoel carefully removed, and the viscera separated to allow the escape of the cysticercoids. The cysticercoids were found in both the thoracic and abdominal regions of the beetles, but more predominately in the latter. Some of the cysticercoids were free in the haemocoele. It was necessary to separate others from the viscera, especially the Malpighian tubules. With the use of a dissecting microscope cysticercoids could be easily seen and recovered with a small-mouthed pipette, often in large numbers. Results of these experiments are summarized in Table 4.

With these procedures for feeding mice and beetles, infections, when once established, could be carried on for several generations.

Histological Procedures

When an infected mouse was autopsied, several adult worms were reserved for slide preparations. A few cysticercoids, for histological study, were set aside after each beetle was dissected. Both whole mounts and sections were prepared when there was sufficient material available. The following procedures were used.

Whole mounts: Adults were relaxed by placing them on a slip of filter paper in a petri dish and flooding them with a warm 10 per cent

solution of ethyl alcohol. The dish and its contents were kept warm over an alcohol lamp. After approximately 30 minutes, most of the alcohol was drawn off and hot AFA was added to fix the worms. The use of filter paper was helpful in preventing the contraction of the worm when the fixative was added. Cysticercoids were fixed in AFA immediately after their removal from a beetle. Whole mounts of adults and cysticercoids were stained, using Delafield's haematoxylin or Mayer's HCl-carmine stains. The use of glass chips as supports for cover slips in whole mount preparations of stained cysticercoids prevented cover slip pressure from flattening the specimens.

Sections: Adults reserved for sectioning were not relaxed before adding Bouin's fixative. Cysticercoids were fixed in AFA and were lightly stained with an alcoholic solution of eosin before embedding, to facilitate finding them in the paraffin. Both adults and cysticercoids were embedded in paraffin for sectioning and were cut at 10 microns. Stains used were Heidenhain's iron haematoxylin, Mayer's acid haemalum, Delafield's hematoxylin, and Heidenhain's "azan" triple stain.

Irradiation Procedures

Cysticercoids and gravid proglottids were irradiated for these studies. All irradiations were conducted with the same x-ray apparatus, at a setting of 250 PKV, 15 milliamperes, and utilizing filters of 1/2

mm. copper and 1 mm. aluminum at a distance of 35 cm. T. S. D. The 1/2 value layer, or quality of irradiation, was 1. 3 cm. Cu. One hundred twenty roentgen units per minute were emitted. The material to be x-rayed was placed in small plastic boxes, approximately 5x2x2 cm., several of which were then arranged on a plywood tray. X-rays passed through plastic and plywood so that an increase in the total amount of irradiation through deflection could be avoided.

The plastic boxes were two-thirds packed with absorbent cotton. Three layers of filter paper were placed on top of the cotton, and mammalian saline used to saturate the cotton and moisten the paper. The material to be x-rayed was then placed on the filter paper. It was found by experimentation that cysticercoids, less resistant than proglottids, remained viable for at least two hours, if the boxes were kept tightly closed except during the period of irradiation. This provided sufficient time not only for x-ray procedures, but also for subsequent feeding of irradiated materials to laboratory-reared mice. After irradiation, the sheet of filter paper containing the irradiated materials was placed in a Syracuse watch glass filled with mammalian saline and gently shaken. Irradiated proglottids were then fed to beetles. With the aid of a dissecting microscope, irradiated cysticercoids were gathered and fed to mice by methods already described.

RESULTS

Feeding Experiments with Mice

As previously indicated, a series of experiments was conducted in an effort to ascertain if it might be possible to predict with some degree of accuracy the number of adult worms which would develop, if the direct cycle of development of Hymenolepis nana were experimentally employed. Certain of these experiments dealt with the feeding of gravid proglottids, and some involved the feeding of eggs. All the white mice used as definitive hosts in these experiments were laboratory-reared.

Results of experiments involving the feeding of gravid proglottids to white mice are summarized in Table 1. Proglottids fed to mice (10 to 14 weeks of age) varied in number from 3 to 10 and mice were sacrificed from 10 to 20 days post-feeding. Reference to Table 1 indicates that approximately 75 per cent of the mice did not become infected when this method of development was employed. Furthermore, of the mice which became infected, no relationship could be shown between the number of proglottids fed and the number of adult worms recovered. Following the feeding of three proglottids, for example, sometimes only a single worm, sometimes as many as eight well-developed adults were found. With increased numbers of proglottids fed, no corresponding increase in number of adults secured could be detected.

Table 1. Summary of experiments involving the feeding of gravid proglottids of Hymenolepis nana to white mice

Proglottids fed immediately after removal from definitive host			
Number of proglottids fed	Age of mouse (in weeks)	Mouse killed post-feeding (in days)	Number of adult worms recovered
3	12	10	0
3	12	10	0
3	12	10	1
3	12	10	0
3	12	10	0
3	12	10	8
3	12	10	0
3	12	10	0
3	12	10	0
7	14	15	4
8	11	15	0
9	11	15	0

Proglottids incubated at room temperature				
Number of proglottids fed	Hours incubation	Age of mouse (in weeks)	Mouse killed post-feeding (in days)	Number of adult worms recovered
3	24	13	18	0
4	24	13	18	2
5	24	14	18	0
6	24	14	18	0
7	24	13	18	0
7	24	11	20	0
8	24	12	20	0
9	24	12	20	0
10	24	12	20	6
4	40	14	18	0
8	40	14	18	1
9	40	14	18	0
10	40	14	18	0

Proglottids incubated in refrigerator				
Number of proglottids fed	Hours incubation	Age of mouse (in weeks)	Mouse killed post-feeding (in days)	Number of adult worms recovered
4	12	10	10	1
6	12	10	10	0

Preliminary incubation of the proglottids at room temperature, or their refrigeration before being fed to mice apparently had little effect on stimulating the onchospheres to hatch or to complete their development in mice. Mice killed 10 days post-feeding contained approximately the same number of worms as did those which were not killed until 20 days post-feeding. Fully developed worms were recovered after 10 days as well as after 20. The percentage of gravid worms recovered, consequently, does not increase with a longer time interval between feeding and the examination of the host animal.

A summary of experiments involving the feeding of eggs of H. nana to white mice is presented in Table 2. Using this method of infecting mice, approximately 50 per cent of them developed mature tapeworms, in contrast to the 25 per cent when proglottids were employed. Mice used in these experimental feedings were 10 to 12 weeks old, and were sacrificed 10 to 15 days post-feeding. In those instances where hosts became infected, the number of worms recovered from a single mouse was frequently greater in relationship to the number of eggs fed, than the number of worms recovered when proglottids were fed. However, as Table 2 indicates, no consistency could be demonstrated between the number of eggs fed and the number of worms recovered. Incubation of eggs at room temperature for 24, 40, or 72 hours prior to feeding apparently had no stimulatory effect upon development of cysticercoids.

Table 2. Summary of experiments involving the feeding of Hymenolepis nana eggs to white mice

Eggs fed immediately after recovery			
Number of eggs fed	Age of mouse (in weeks)	Mouse killed post-feeding (in days)	Number of adult worms recovered
3	10	10	0
14	10	10	0
20	12	10	3
31	12	10	0

Eggs incubated at room temperature				
Number of eggs fed	Hours incubation	Age of mouse (in weeks)	Mouse killed post-feeding (in days)	Number of adult worms recovered
5	24	12	10	0
8	24	12	10	4
15	24	12	10	1
20	24	12	10	12
25	24	12	10	0
25	24	10	10	2
3 proglottids[a]	24	11	10	0
4 proglottids	24	11	10	1
5 proglottids	24	11	10	1
6 proglottids	24	11	10	1
7 proglottids	24	11	10	5
10	40	12	10	2
7 proglottids	40	12	10	16
10 proglottids	40	11	10	4
9	72	11	10	0
25	72	12	10	0
25	72	12	10	2

Eggs incubated in refrigerator				
Number of eggs fed	Hours incubation	Age of mouse (in weeks)	Mouse killed post-feeding (in days)	Number of adult worms recovered
4 proglottids	24	10	15	1
4 proglottids	24	10	15	0
6 proglottids	24	10	15	0

[a]Gravid proglottids were placed in saline, the body walls ruptured to permit release of eggs. The suspension was centrifuged, the supernatant carefully removed, and the entire mass of eggs fed to a mouse.

The lack of uniformity of results obtained with the use of proglottids and of eggs as means of infecting mice led to the use of the indirect life cycle in which white mice were fed cysticercoids obtained from the beetle, Tenebrio molitor. All cysticercoids employed in these experiments were experimentally reared in beetles in the laboratory. A summary of these experiments is presented in Table 3, reference to which indicates that this method is by far the most effective one in obtaining adult worms. Approximately 82 per cent of the cysticercoids fed to non-pregnant white mice (two to four months of age) could be expected to develop to maturity. By accident, two pregnant white mice were fed cysticercoids. In both instances, only a single adult tapeworm was recovered. These results support the theory that a partial immunity develops during pregnancy of the host animal. In another experiment, a mouse having previously been fed eight cysticercoids, was found to harbor only two adult worms. Inasmuch as the mouse was not pregnant, and was within the age limit for susceptibility, and since cysticercoids employed appeared viable, the most plausible explanation is one involving biological variability. On the other hand, another mouse of the same age (almost four months old) which had been fed three cysticercoids harbored three adults. A third mouse, which had been fed 12 cysticercoids, contained ten adults at autopsy. Despite this inconsistency of the results, the experiments indicate that the use of cysticercoids is the most satisfactory means of

Table 3. A summary of experiments involving the feeding of Hymenolepis nana cysticercoids to white mice

Number of cysticercoids fed	Age of mouse (in weeks)	Mouse killed post-feeding (in days)	Number of adult worms recovered	Percentage recovery
3	12	12	2	66 %
4	12	12	3	75
12	15	11	10	83
3	15	11	3	100
8	15	12	2	25
5	13	10	4	80
3	13 (pregnant)	10	1	33
5	14	10	4	80
9	14	10	8	88
16	14	10	14	87
8	14	10	8	100
21	14	10	15	71
13	14	10	9	69
7	14 (pregnant)	10	1	14
4	11	10	2	50

obtaining fairly constant numbers of gravid worms.

Feeding Experiments with Beetles

Since the most reliable method of predicting the number of adults expected to develop in experimentally infected mice was one utilizing the indirect life cycle, techniques were developed for feeding proglottids to beetles and for recovering infective cysticercoids. A summary of this work is presented in Table 4. Tenebrio molitor was found to be a suitable host, easily maintained in the laboratory and readily infected.

Table 4. Summary of experiments involving the feeding of Hymenolepis nana proglottids to Tenebrio molitor

Force-feeding technique

Number of beetles exposed	Number of proglottids fed	Age of cysticercoids (in days)	Results		
			Positive	Negative	Died
6	2-4	15	---	1	--
		16	---	1	--
		18	2(1, 7)[a]	--	2
10	2-4	19	3(5, 8, 8)	5	2
10	3-6	17	2(3, 12)	8	--
10	6-8	16	2(1, 6)	4	4
5	6-8	17	3(6, 6, 10)	2	--
20	6-8	16	6(1, 1, 3, 6, 8, 9)	9	5
15	8-10	14	4(1, 1, 9, 10)	8	3

Apple-feeding technique

Number of beetles fed	Age of cysticercoids (in days)	Results		
		Positive	Negative	Died
6	14	3(3, 4, 6)[a]	3	--
10	19	3(8, 14, 14)	7	--
10	17	5(3, 5, 11, 11, 19)	3	2
10	17	---	7	3
26	13	10(1, 2, 5, 9, 13, 17, 21, 21, 21, 24)	14	2
14	14	4(1, 4, 8, 13)	7	3
15	14	12(1, 2, 3, 4, 5, 5, 7, 8, 18, 19, 33, 86)	3	--
20	14	12(1, 2, 2, 2, 2, 2, 4, 5, 8, 10, 10, 10)	6	2

[a]Numbers in parentheses indicate actual number of cysticercoids recovered in each positive infection.

Two methods of feeding were used, details of which have already been outlined in a previous section. One method, which might be termed "force-feeding" is a time-consuming one. The second method ("apple-feeding") is rapid and simple. The number of infected beetles resulting from the force-feeding method is not so large as the number of infected ones resulting from use of the second method. Furthermore, beetles which become infected as a result of force-feeding generally do not contain as many cysticercoids as do those beetles which are apple-fed. One of the difficulties inherent in the apple-feeding technique is that there is no reliable method of determining the number of proglottids ingested by an individual beetle. As indicated in Table 4, there is no relationship between the number of proglottids force-fed to a beetle (two to ten proglottids in these experiments) and the number of cysticercoids subsequently produced. A similar relationship existed in the experiments involving the feeding of proglottids to mice.

Regardless of the feeding techniques employed, however, well-developed cysticercoids were recovered from the haemocoels of the intermediate hosts after 13 to 19 days post-feeding. With the force-feeding technique the greatest number of cysticercoids recovered was 12. As many as 86 fully developed cysticercoids were recovered from one beetle, however, when the apple-feeding method was employed.

Irradiation Experiments

Irradiation of proglottids

Two extensive experiments were conducted in an effort to determine the possible effects of x-irradiation on gravid proglottids as well as on cysticercoids of Hymenolepis nana.

Gravid proglottids were exposed to varying amounts of x-irradiation ranging from 60 to 3600 roentgens. Following irradiation, the proglottids were fed to laboratory-reared beetles (Tenebrio molitor) and after 14 days the beetles were examined for the presence of cysticercoids. Results of these two experiments are indicated in Table 5. In some cases, beetles were found to harbor no cysticercoids, even those beetles which had been fed proglottids having received the lowest amount of irradiation. The fact that a beetle was negative for Hymenolepis, however, cannot be considered as convincing evidence that irradiation was responsible for failure of cysticercoids to develop, since only two experiments were conducted. In control work, the likelihood that a beetle would become infected when fed proglottids was shown to be quite low (see Table 1). It is of interest to note that even at high dosage levels, such as 3600 roentgens, eggs from proglottids fed to beetle hosts did develop to well-formed cysticercoid stages. The amount of irradiation administered to proglottids, then, apparently had no decisive effect on the number of cysticercoids recovered.

Table 5. Summary of experiments involving irradiation of gravid proglottids

Experiment 1

Amount of irradiation	1st larval generation		1st adult generation			2nd larval generation
	Number of cysticercoids recovered from beetles (*T. molitor*)	Number cysticercoids fed to white mice	Number adult worms recovered	% recovery	No. gravid proglottids fed to beetle	No. cysticercoids recovered 14 days post-feeding
120r	0					
120r	5	3	3	100%	5-6	416
620r	7	5	5	100	5-6	42
620	0					
620	0					
1000	0					
1000	0					
1500	1					
2000	0					
2000	12	6	1	16		

Table 5. (con't.)

Experiment 2

Amount of irradiation	1st larval generation: Number of cysticercoids recovered from beetles (T. molitor)	1st larval generation: Number cysticercoids fed to white mice	1st adult generation: Number adult worms recovered	1st adult generation: % recovery
60r	0			
60r	0			
120r	60	6	3	50%
		6	4	66
120r	0			
480r	0			
960r	98	6	6	100
		6	2	33
		6	6	100
		6	0	0
		6	6	100
		6	6	100
1200r	0 (host died)			
1200r	24	6	1	33
		6	4	66
1400	0			
1800	0			
2040	0			
2400	0			
2400	0			
3000	0			
3000	0			
3600	0			
3600	5	3	2	66

Whole mount preparations of certain of the cysticercoids recovered from beetles indicate that no morphological abnormalities are apparent. As shown on Table 5, certain cysticercoids of the first larval generation were fed to laboratory-reared white mice in order to obtain the first adult generation. In almost every instance, adult worms did develop, but the expected survival rate of 82 per cent, as obtained in control experiments, did not always occur. It would appear that no relationship exists between the amount of radiation received by the original proglottid and the percentage survival of the first adult generation. The inability to control the amount of irradiation received by an individual onchosphere encased within the proglottid may account for the inconclusive results of these experiments. Microscopic examination of whole mounts and serial sections of adult worms of the first generation indicated that morphologically they appeared normal in all respects. They demonstrated no sexual immaturity as a result of previous exposure to irradiation, and all gravid proglottids contained eggs which seemingly appeared normal.

In the first of the two experiments involving irradiation of proglottids, proglottids of the first adult generation were fed to beetles and a second larval generation obtained. These cysticercoids of the second generation had developed from eggs within proglottids having originally been exposed to dosages of 120r and 620r, respectively. Cysticercoids developed

within the beetles were recovered in quantity 14 days later. These larvae, too, appeared normal in all respects.

No experiments were conducted in which eggs removed from the proglottids were directly exposed to irradiation. Brillhart (1958), however, working on a related species of hymenolepid, namely H. diminuta, found that exposure of eggs of this species to a dosage of 15,000 r resulted in their failure to develop, and that cysticercoids developing from eggs exposed to 10,000 r were limited in number and were abnormal in appearance.

Irradiation of cysticercoids

Cysticercoids obtained from the body cavities of experimentally reared beetles were exposed to x-irradiation ranging from 120r to 4500r (see Table 6). Two experiments of this type were conducted, both of which involved, in part, the subsequent feeding of the cysticercoids to mice so as to obtain the first adult generation. No adult worms were recovered when previous irradiation of the cysticercoids had exceeded 3500r. Proglottids from adult worms developed after lower rates of irradiation were removed from the mammalian host, then were fed to beetles in order to secure the second-generation cysticercoids.

In the first of the two experiments summarized in Table 6, radiation dosages varied from 120r to 2240r, and in almost every case, adult worms were recovered. In the second experiment, where dosages varied from

Table 6. Summary of experiments involving irradiation of cysticercoids

Experiment 1

Amount of irradiation	Number of irradiated cysticercoids fed to white mice	1st adult generation			2nd larval generation
		No. of worms recovered 10 days post-feeding	% recovery	No. proglottids fed to beetle	Number of cysticercoids recovered 14 days post-feeding
120r	4	1	25%	5-6	0
120r	6	4	66	5-6	60
240r	5	5	100	5-6	126
240r	5	5	100	5-6	0
500r	8	1	12	5-6	2
500r	6	0	0		
720r	8	3	37	5-6	100
720r	9	4	44	5-6	0
1000r	6	3	50	5-6	0
1200r	16	5	31	5-6	51
1500r	11	7	63	5-6	0
2000r	5	1	20	5-6	0
2240r	9	1	11	5-6	0

Table 6. (con't.)

Experiment 2

Amount of irradiation	Number of irradiated cysticercoids fed to white mice	1st adult generation			2nd larval generation
		No. of worms recovered 10 days post-feeding	% recovery	No. proglottids fed to beetle	Number of cysticercoids recovered 14 days post-feeding
500r	6	5	83%	5-6	28
1000r	5	1[a]	20	5-6	45
1500r	4	3	75	5-6	135
2000r	5	1	20	5-6	0
2250r	5	1	20	5-6	0
2750r	7	1	14	5-6	0
3000r	3	0	0		
3500r	8	1	12	5-6	0
3750r	5	0			
4000r	3	0			
4250r	3	0			
4500r	7	0			

[a]Pregnant mouse.

500 to 4500r, a rather definite relationship could be noted between the amount of irradiation received by the cysticercoids and the number of adults subsequently developed. This was evidenced by the fact that above 3500r, no adult worms were found when the mice were autopsied.

Of considerable interest in both experiments were the results obtained when gravid proglottids of the first adult generation were fed to beetles. The intermediate hosts in such instances failed to acquire hymenolepid infections if the original exposure of cysticercoids exceeded 1500r. At levels above 1500r, consequently, sterility of the second larval generation resulted. In the second experiment, it was clearly shown that at dosages of 2000r or more, the number of second-generation cysticercoids dropped precipitously. Reference to Table 6 (Experiment 2), for example, shows that at 1500r, cysticercoids developed into adult worms, and that cysticercoids of the next generation were found in abundance in the beetle intermediate host. However, cysticercoids exposed to irradiation varying from 2000r to 3500r, even though they developed into adult worms in limited number, were never able to produce cysticercoids of the following generation.

Worms of the first adult generation recovered in both experiments showed no morphological abnormalities when studied microscopically. Those which were unable to produce cysticercoids of the second generation possessed, nonetheless, gravid proglottids containing eggs which

appeared entirely normal. Similarly, cysticercoids of the second larval generation were apparently normal in every respect when examined microscopically.

DISCUSSION

Although previous investigators have, for the most part, used eggs and proglottids as materials for establishing Hymenolepis nana infections in mice, the studies here reported indicate that cysticercoids are far more suitable. Experimentation with all stages showed that adult worms do not develop in any predictable number when mice are fed eggs or proglottids. With cysticercoids, however, a more precise estimate of the parasite burden can be made.

Shorb (1933) calculated the expected percentage of development of H. nana eggs in the final host as follows: using rats as definitive hosts, 0.0002 to 0.429 per cent; using mice, 0.133 to 1.08 per cent. These figures indicate a rather large range and the number of worms expected to develop is much too low to be of practical value in work of this type. Shorb points out, too, that eggs once ingested by a definitive host may be subject to a number of factors influencing their development. For example, they may pass through the body unaltered, or they may be digested by the host because of their non-viability. Even if the oncho-spheres hatch, the larvae may penetrate the intestinal villi and reach abnormal sites where development cannot be completed. Moreover, if an adult worm is produced, it may be unable to maintain itself in the intestine. All these factors influence the number of worms which might

be expected to develop when eggs are used in establishing infections.

Hollaender, Jones and Jacobs (1940), working with the pinworm, Enterobius vermicularis, developed the "R-C" survival ratio to determine the percentage of eggs expected to survive but which were inactivated due to previous ultra-violet irradiation. Applying their procedures to H. nana, the following method could be employed:

$$\frac{\text{Number of developed cysticercoids}}{\text{Number of irradiated eggs injected}} = \text{Ratio of survival of irradiated eggs (R)}$$

$$\frac{\text{Number of cysticercoids in control}}{\text{Number of eggs injected in control}} = \text{Ratio of survival of control eggs (C)}$$

$$\frac{R}{C} = \text{Ratio of survival of irradiated eggs expressed in per cent}$$

The remaining percentage (i. e., of 100 per cent) represents the per cent of viable eggs inactivated by irradiation. Applying this R-C ratio to Shorb's figures (0.1% to 1.0% for expected percentage development of H. nana eggs in mice) the possible range that would be obtained between the survival ratio in controls (C) and survival ratio in test animals (R) is too great to be of value in experiments concerned with egg development. The 82 per cent survival of cysticercoids shown by my studies, on the other hand, appears to be fairly constant.

Another advantage resulting from the use of cysticercoids as the infective stage relates to their size. A large number of cysticercoids may be isolated in a small quantity of fluid, and these are visible to the naked eye when seen against a dark background. With a pipette,

cysticercoids in known quantity may be readily offered to water-starved mice.

Although experimental evidence showed that 14 days are sufficient for the development of cysticercoids in beetles, those left in the intermediate host for as long as 19 days were found to be viable when fed to a mouse. How long the cysticercoids may remain infective within the haemocoel of a beetle was not determined, nor was the viability of cysticercoids less than 13 days old investigated.

The use of Tenebrio molitor as an intermediate host was found to be very satisfactory. This beetle is large enough to handle easily, is fairly susceptible to H. nana infections, and is very readily maintained in the laboratory. Experimentation seemed to indicate that younger beetles are more susceptible to infection than are older ones. The possibility of age resistance as a factor in infectivity would provide an interesting avenue of investigation for further studies on this host.

For irradiation studies, the cysticercoid stage again was of greater usefulness. If one is concerned with acquiring data relative to the amount of irradiation needed to kill, to sterilize, or to otherwise affect an organism, it is essential to determine, insofar as possible, the precise amount of irradiation received by that organism. When proglottids are irradiated, such factors as the thick cuticle, extensive parenchymal musculature and numerous layers of eggs surrounded by

thick shells must be taken into account. Absorption of x-rays by the body wall is difficult to determine quantitatively, and the amount of irradiation received by the eggs in the lower half of the proglottid cannot readily be compared with the amount of irradiation received by those eggs in the upper half. Egg membranes surrounding the onchosphere may also absorb some of the rays. Using cysticercoids for irradiation studies, on the other hand, there is little doubt that each organism receives the same amount of irradiation.

The results obtained in the experiments herein reported, and the lack of morphological abnormalities of individuals from parasite generations developed from irradiated cysticercoids and proglottids, leads me to conclude that within the range of irradiation here used, any effects of x-rays upon H. nana may be of a genetic nature. Other workers, too, dealing with the effects of irradiation on various parasites, have reported the development of normal-appearing adults that were sterile. Schwartz (1921), working with Trichinella spiralis, found that injury from x-rays was not visible structurally or functionally in juveniles, but became apparent only when the juveniles reached a suitable host in whose intestine they did not develop, even though normally they would have continued their development there. Emmett (1950) x-rayed Trypanosoma cruzi and found these organisms remain viable and morphologically unchanged, but noted that a definite decrease in infectivity had been

induced. Alicata (1951) exposed juveniles of Trichinella spiralis to x-rays and found that 10,000r would permit juveniles to develop into morphologically normal adults, but that these adults could not produce young. Semrad (1937) found that juveniles of T. spiralis freed from muscle survived exposures of 1200r and continued their development in the intestine of rats but did not produce young. Gould, van Dyke, and Gomberg (1953) found that 3500r permitted maturation of T. spiralis juveniles to sterile adults which appeared normal in every respect.

Recent studies by certain workers, on the other hand, indicate that at higher levels of x-irradiation, definite morphological variations result. Schiller (1957) irradiated eggs of H. nana with dosages ranging from 5000 to 40,000r and reported that the resultant mutations in adult H. nana were directly proportional to the roentgen dose. Data of Schiller indicate 30,000r as the amount of x-irradiation needed to produce sterile adults. In my experiments with cysticercoids, however, 1500r were shown to be sufficient to cause sterility of adult worms. The considerable difference between these two extremes must be associated with the nature of the material x-rayed. Brillhart (1958), working with H. diminuta, was able to recover only four abnormal cysticercoids, two each from two of 30 infected rats; these cysticercoids had developed from eggs exposed to 10,000r.

These facts indicate, then, that cysticercoids are far less resistant

to the effects of radiation than are any of the other stages normally employed in studies of this nature. It is of interest that parasitic organisms such as the tapeworm are capable of withstanding far greater amounts of radiation than their mammalian hosts. With the techniques suggested in these studies, it would be of considerable interest to study the effect on host resistance of tapeworms such as H. nana when the host itself has undergone exposure to limited amounts of x-irradiation. Possibly increased susceptibility to parasitism might be shown. The fact that as many as 416 cysticercoids were recovered from the haemocoel of one beetle, fed proglottids from adult worms developed from gravid proglottids irradiated at 120r (see Table 5), suggests that, at low levels of irradiation, the possibility of parasite development is enhanced. Also, as shown on Table 6, as the level of irradiation of proglottids from 500r to 1500r increases, there appears to be a progressive increase in number of cysticercoids of the second larval generation. These results suggest the desirability of additional studies on these aspects of host-parasite relationships.

SUMMARY AND CONCLUSIONS

1. The use of proglottids or eggs of the dwarf tapeworm, Hymenolepis nana for infecting laboratory-reared mice is of less value than is the use of cysticercoids. Using proglottids and eggs, no assurance can be given that a definite number of cestodes will develop.
2. Incubation of proglottids and/or of isolated eggs at room temperatures, or their refrigeration before feeding them to mice, has no effect on numbers of adult worms developing in the definitive host.
3. No apparent relationship exists between the number of gravid proglottids or eggs fed to a mouse and the number of adult worms subsequently developed.
4. When cysticercoids are fed to laboratory-reared white mice, approximately 82 per cent can be expected to develop into adult worms ten days post-feeding.
5. The mealworm beetle, Tenebrio molitor, serves as a suitable intermediate host for H. nana and may be infected either by force-feeding (placing proglottids on the mouth parts of an immobilized insect) or by an "apple-feeding" technique whereby beetles feed at will on small pieces of apple covered with gravid proglottids.
6. A period of 14 days is sufficient time for development of cysticercoids within the haemocoel of the invertebrate host.

7. No relationship can be noted between the number of gravid proglottids fed to a beetle and the number of cysticercoids expected to develop. With the apple-feeding technique, however, larger numbers of cysticercoids are obtainable.

8. Gravid proglottids of experimentally-reared adult worms were exposed to varying amounts of x-irradiation ranging from 60 to 3600 roentgens. Eggs from these proglottids, when fed to beetles, produced normal cysticercoids. Adult worms of the following generation, too, were normal in appearance.

9. Cysticercoids were exposed to x-irradiation ranging from 120 to 4500 roentgens. No adult worms developed from cysticercoids whose irradiation dosage had exceeded 3500r.

10. If cysticercoids are exposed to more than 1500r, but less than 3500r, the adult worms developing from those cysticercoids produce only sterile eggs.

11. No morphological alterations could be detected in those cysticercoids which survived the initial radiation, nor were their progeny abnormal.

12. Within the range of irradiation used in these experiments, the effects of x-rays on H. nana are of a genetic nature.

13. The fact that cysticercoids are far less resistant to the effects of x-irradiation than are either proglottids or eggs, suggests the desirability of the use of cysticercoids in further studies on host-parasite relationships.

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